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Phenylalanine hydroxylase and isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase in relationship to the phylogenetic position of *Pseudomonas acidovorans* (*Ps.* sp. ATCC 11299a)

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Abstract. The evolution of aromatic amino acid biosynthesis and its regulation is under study in a large assemblage of prokaryotes (Superfamily A) whose phylogenetic arrangement has been constructed on the criterion of oligonucleotide cataloging. One section of this Superfamily consists of a well defined (rRNA homology) cluster denoted as Group III pseudomonads. *Pseudomonas acidovorans* ATCC 11299a, a Group III member, was chosen for in-depth studies of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, the initial regulatory enzyme of aromatic biosynthesis. This strain is of particular interest for evolutionary studies of aromatic metabolism because it possesses phenylalanine hydroxylase, an enzyme whose physiological role and distribution among prokaryotes is largely unknown. Although *P. acidovorans* ATCC 11299a has been of uncertain identity, we now establish it unambiguously as a species of *acidovorans* by virtue of its 87% DNA homology with *P. acidovorans* ATCC 15668 (type strain). This result conformed with enzyme patterning studies which placed ATCC 11299a into pseudomonad Group IIIa, a subgroup containing the *acidovorans* species. Crude extracts of Group III pseudomonads had previously been shown to share, as a common group characteristic, sensitivity of DAHP synthase to feedback inhibition by either L-tyrosine or L-phenylalanine. Detailed studies with partially purified preparations from strain ATCC 11299a revealed the presence of two distinct regulatory isozymes, DAHP synthase-phe and DAHP synthase-tyr. DAHP synthase-tyr is tightly controlled by L-tyrosine with 50% inhibition of activity being achieved at 4.0 μ*M* effector. DAHP synthase-phe is inhibited 50% by 40 μ*M* L-phenylalanine and exhibits dramatic changes in levels of activity, as well as chromatographic elution patterns, in response to dithiothreitol. This two-isozyme pattern of DAHP synthase has not been described previously, although it may prove to be widespread.

Key words: Phylogeny – Pseudomonads – Aromatic biosynthesis – DAHP synthase – Phenylalanine hydroxylase

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Abbreviations. DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; E4P, D-erythrose-4-phosphate; PEP, phosphoenolpyruvate; DTT, dithiothreitol; BSA, fraction V bovine serum albumin

The modern molecular approach of oligonucleotide cataloging (Fox et al. 1980) generates phylogenetic-based microbial classifications that may differ drastically from traditional groupings found in Bergey's manual. Given this new access to phylogenetic lineages, the once unapproachable objective of tracing the evolution of particular biochemical pathways becomes feasible (Jensen 1984).

The multi-branched pathway for the biosynthesis of aromatic amino acids is diverse in nature, exhibiting variability with respect to pathway routing, cofactor specificity of dehydrogenases, and allosteric control patterns (Byng et al. 1982, 1983b). The first enzyme in aromatic biosynthesis, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, has been extensively studied in this regard, and a variety of distinctive patterns are known (Byng et al. 1982). Early-pathway allosteric control is usually observed to be in force at the level of DAHP synthase, even when late-pathway control is weak or absent; a rationale has been presented to account for evolution of early-pathway regulation as an ancient event (Jensen and Hall 1982).

Isozymic species of DAHP synthase that are subject to different feedback inhibitor signals are well known. In Superfamily B prokaryotes a feasible evolutionary scenario is emerging (Byng et al. 1983a) whereby the postulated Superfamily ancestor already possessed two isozymes, DAHP synthase-tyr and DAHP synthase-trp, that were feedback inhibited by L-tyrosine and L-tryptophan, respectively. This arrangement has been conserved in Group I pseudomonads (Byng et al. 1983a). The separate evolutionary trails of Group V pseudomonads and enteric bacteria have led to loss of DAHP synthase-tyr and addition of the L-phenylalanine-regulated isozyme, DAHP synthase-phe, respectively. Concrete evidence is appearing (Davies and Davidson 1982) in support of the thesis that DAHP synthase isozymes probably originated by gene duplication followed by specialization of allosteric control (Jensen 1976).

Within the Superfamily A assemblage of prokaryotes (Byng et al. 1983b), the only published reports of DAHP synthase allostery are for the two rRNA homology clusters of pseudomonad bacteria denoted as Group II and Group III. Both groups exhibited allosteric sensitivity to L-tyrosine or to L-phenylalanine when DAHP synthase was assayed in crude extracts (Whitaker et al. 1981a). Feedback sensitivity to multiple effectors may either reflect the existence of differentially controlled isozymes or of one enzyme bearing multiple allosteric binding sites (Jensen and Rebello

1970). Partial purification will be required to distinguish between the latter possibilities, and the present account of the Group III arrangement for DAHP synthase is the beginning of a systematic evaluation aimed at deductions about the evolution of DAHP synthase regulation in Superfamily A members.

Pseudomonas acidovorans ATCC 11299a is noted for its ability to form L-tyrosine from L-phenylalanine via the enzyme phenylalanine hydroxylase (Guroff and Ito 1963), and is thus of considerable interest from the standpoint of the overall impact of this capability upon aromatic amino acid metabolism. The exact taxonomic position of ATCC 11299a has been, however, somewhat dubious. Since its original isolation as a colonial variant of S.H. Hutner's strain Tr-6, or ATCC 11299 (Stanier et al. 1951), the organism has been referred to at different times as *Pseudomonas* sp. (Guroff and Ito 1963), *P. acidovorans* (Rosenfeld and Feigelson 1969), and *Comamonas terrigena* (Guroff et al. 1970). Currently, this pseudomonad is listed in the 1982 catalog of strains (ATCC) as a strain of *P. acidovorans*. Once we rigorously affirmed the identity of strain ATCC 11299a as a strain of *acidovorans*, we studied it as a representative of Group III pseudomonads in order to develop information that may eventually contribute insight into the possible interplay between the biosynthetic pathway and what is probably a catabolic enzyme (i.e., phenylalanine hydroxylase). It is possible that phenylalanine hydroxylase can assume at least a secondary role in L-tyrosine biosynthesis under some nutritional conditions.

It is interesting that studies having the same rationale were done almost 20 years ago (Cerutti and Guroff 1965), but prior to the appreciation of the biochemical diversity that exists for L-phenylalanine and L-tyrosine biosynthesis (Byng et al. 1982).

Materials and methods

Microbiological information

Pseudomonas acidovorans (*Pseudomonas* sp.) ATCC 11299a was originally obtained from the American Type Culture Collection. The prior listing (1982 catalog of strains, ATCC) of this organism, as well as other *P. acidovorans* strains ATCC 9355, 11299b, and 13751), as strains of *Comamonas terrigena* raises the possibility that *P. acidovorans* and *C. terrigena* may be synonomous. DNA homology studies between the type strain of *P. acidovorans* (ATCC 15668) and *C. terrigena* (ATCC 8461) would answer this question.

Stocks of *P. acidovorans* ATCC 11299a were maintained on Difco nutrient agar slants at room temperature. Cells were grown at 22° C in a modified version of the minimal salts medium of Guroff and Ito (1963) [containing (per liter of glass-distilled H_2O): KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; $(NH_4)_2SO_4$, 2.0 g; succinic acid, 2.0 g; L-proline, 0.05 g]. Media were adjusted to pH 7.0 with KOH and, when necessary, solidified with 1.5% (w/v) Difco agar. Liquid cultures were grown to late exponential phase either on a New Brunswick rotary shaker at 150 rpm, or for large volumes (16 l), in 20-l carboys as previously described for *P. aeruginosa* (Whitaker et al. 1982). A listing of other organisms used for DNA homology experiments can be found in Table 2. Culture conditions for these species have been described (Byng et al. 1980).

DNA homology determinations

High molecular weight DNA was isolated by the method of Marmur (1961). The preparations were stored at −20° C in 0.1X SSC−1.0 m*M* Hepes (pH 7.0). SSC is 0.15 *M* NaCl and 0.015 *M* sodium citrate (pH 7.0). Hepes is sodium N-2-hydroxyethylpiperazine-N-2-ethanesulfonate. The DNA preparations were fragmented for use in homology experiments by three passages through a French pressure cell at 1.1×10^8 Pa. The DNA was denatured by heating in a boiling water bath for 5 min. After cooling in an ice-water bath, the DNA preparations were centrifuged at $12{,}000 \times g$ for 15 min. The concentration of each preparation was then adjusted to 0.4 mg/ml, and preparations were stored at −20° C. Labelled DNA was prepared by iodination (Selin et al. 1983). The specific activity of the labelled preparations was 2×10^6 cpm/μg. DNA homology values were determined using the S1 nuclease procedure (Johnson 1981). The reassociation vials each contained 10 μl of labelled DNA (0.1 μg), 50 μl unlabelled DNA (20 μg), 25 μl of 5.28 *M* NaCl−1.0 m*M* Hepes (pH 7.0), and 25 μl of deionized formamide. This results in a sodium ion concentration equivalent to 6X SSC and a formamide concentration of 23%. The vials were incubated at 63° C for 24 h, which is 25° C below the Tm of the native DNA in this buffer system.

Preparations of extracts of P. acidovorans ATCC 11299a for enzyme assay

Crude extracts were prepared at 4° C as previously described (Byng et al. 1983c). Extract buffer was either 40 m*M* potassium phosphate, pH 7.0 (for hydroxylapatite chromatography) or 50 m*M* potassium phosphate, pH 7.0, containing 1.0 m*M* dithiothreitol [DTT] (for DEAE-cellulose chromatography).

DEAE-cellulose chromatography

Crude extract containing 125 mg of protein was applied to a 1.5 × 20 cm Whatman DE-52 column previously equilibrated in 50 m*M* potassium phosphate buffer (pH 7.0) containing 1.0 mM DTT. Protein was washed onto the column with 100 ml of starting buffer, and the column was then eluted with 300 ml of a linear 0 to 0.5 *M* KCl gradient prepared in the same starting buffer. Fractions of 2.2 ml were collected.

Hydroxylapatite chromatography

Crude extract containing 80 mg protein was loaded onto a hydroxylapatite column (20 ml bed volume) equilibrated in 40 m*M* potassium phosphate buffer, pH 7.0. The column was washed with 50 ml of starting buffer, and bound protein was then eluted with 300 ml of a linear potassium phosphate gradient (0.04−0.15 *M*, pH 7.0); 2.2-ml fractions were collected.

Gel filtration

Fractions containing each isozyme band of DAHP synthase that had been fractionated on DEAE-cellulose or hydroxylapatite were pooled (separately), concentrated by Amicon PM-10 ultrafiltration, and applied to a 2.5 × 62.5-cm column of LKB Ultrogel AcA34 which had previously been

Table 1. Enzymological patterning of aromatic amino acid biosynthesis in pseudomonad Groups II and III

Group[a]	ATCC no.	Representative *Pseudomonas* species	Dehydrogenase patterning profile[b]				Dehydratase substrate[c]	
			Prephenate		Arogenate		PPA	AGN
			NAD	NADP	NAD	NADP		
IIa	25609	*P. cepacia*	+	+	[+]	[+]	(+)	+
	10248	*P. marginata*	+	+	[+]	[+]	(+)	+
IIb	10692	*P. solanacearum*	+	+	[+]	[+]	+	+
	27511	*P. pickettii*	+	+	[+]	[+]	+	+
IIIa	15668	*P. acidovorans*	+	+	−	+	(+)	−
	17510	*P. testosteroni*	+	+	−	+	(+)	−
	17724	*P. palleronii*	+	+	−	+	(+)	−
	11299a	*P. acidovorans*	+	+	−	+	(+)	−
IIIb	17505	*P. delafieldii*	+	+	[+]	+	(+)	+
	11228	*P. facilis*	+	+	+	+	(+)	+
IIIc	15946	*P. saccharophila*	+	+	+	+	(+)	+

[a] Enzymological patterning group as previously described (Byng et al. 1980; Whitaker et al. 1981a, b)

[b] As described in Byng et al. (1980). A + represents dehydrogenase activity with the indicated substrate/cofactor combination. Enclosure within brackets indicates that the enzyme activity is sensitive to inhibition by L-tyrosine; otherwise the activity shown is insensitive to inhibition. A − represents no detectable activity. Data for *P. acidovorans* 15668 and *P. acidovorans* 11299a were recently obtained and therefore are not found in Byng et al. (1980)

[c] See Whitaker et al. (1981b). Presence (+) or absence (−) of prephenate (PPA) dehydratase or arogenate (AGN) dehydratase activities. Enclosure within parentheses indicates activation of PPA dehydratase by L-tyrosine. Data for *P. acidovorans* 11299a were not included in Whitaker et al. (1981b)

equilibrated in the same buffer used for the preceding fractionation step. The flow rate was 10 ml/h, and fractions of 1.3 ml were collected. Molecular weight standards used for calibration were: catalase (210,000), aldolase (158,000), BSA (67,000), hen egg ovalbumin (43,000), and chymotrypsinogen A (25,000). The void volume was determined using blue dextran.

Enzyme assays

All reactions were carried out at 37° C under conditions of proportionality with respect to both protein concentration and time. DAHP synthase was assayed as previously described (Jensen and Nester 1966). Standard reaction mixtures contained 50 m*M* potassium phosphate buffer (pH 7.0), 1.0 m*M* D-erythrose-4-phosphate (E4P), 1.0 m*M* phosphoenolpyruvate (PEP), 1.0 m*M* $MnSO_4$ or $CoSO_4$ (if specified), and enzyme in a final volume of 0.2 ml. Substrates and metal-ion salts were prepared in assay buffer and glass-distilled H_2O, respectively. Appropriate controls were included in all assays. Prephenate and arogenate dehydrogenase activities were assayed as described by Byng et al. (1980). Protein concentrations were determined using the method of Bradford (1976).

Biochemicals and chemicals

Amino acids, E4P (sodium salt), PEP (trisodium salt), phenylpyruvate, DTT, protein standards, and Sephadex G-25 were obtained from Sigma Chemical Co. Hydroxylapatite (Bio Gel HTP) was supplied by Bio-Rad. Chorismate, prephenate, and L-arogenate were prepared as previously described (Whitaker et al. 1981a). All chemicals were standard reagent grade.

Results

Enzymological patterning of P. acidovorans 11299a

Since *Pseudomonas acidovorans* 11299a possessed an arogenate/NADP dehydrogenase activity that was completely insensitive to inhibition by L-tyrosine (Table 1), it was assigned to pseudomonad Group III (Byng et al. 1980). This criterion has proven to be highly reliable (Byng et al. 1983b). Subgroup assignments within Group III are based upon enzymatic patterns of one of two dehydratases that may function in L-phenylalanine biosynthesis (Whitaker et al. 1981b). *P. acidovorans* 11299a lacks arogenate dehydratase activity (R. J. Whitaker, unpublished), a feature which places this organism within Group IIIa.

DNA homology studies

Identification of *P. acidovorans* 11299a as a Group IIIa organism remained tentative until nucleic acid hybridization experiments could be carried out (Table 2). *P. acidovorans* (ATCC 11299a) in fact showed 87% DNA homology with the type strain of *P. acidovorans* (ATCC 15668), and therefore should be permanently named as a strain of *P. acidovorans*. It also showed relatively high (17%) DNA homology with *P. testosteroni* (ATCC 17510). This is consistent with findings (Ralston et al. 1972) that strains of *P. acidovorans* and *P. testosteroni* exhibit some (33%) DNA homology with each other, but little or none with other *Pseudomonas* species. Numerous other similarities between *P. acidovorans* and *P. testosteroni* have been noted in the literature (Baumann and Baumann 1978; Byng et al. 1980; Cohen et al. 1969; Jensen et al. 1967; Queener and Gunsalus 1970; Stanier et al. 1966; Wheelis et al. 1967; Whitaker et al. 1981b; Yamada et al. 1982).

Table 2. Percent DNA homology between *Pseudomonas acidovorans* 11299a and representative species of pseudomonad Groups II, III, and IV

rRNA homology Group[a]	Enz. patt. Group[b]	ATCC no.	Species[c]	Avg. % homology[d]
II	IIa	10856	*P. cepacia*	2
		25417	*P. marginata*	7
	IIb	10692	*P. solanacearum*	3
		15958	*P. pyrrocinia*	2
	IIx	17697	*A. eutrophus*	5
III	IIIa	11299a	*P. acidovorans*	99
		15668	*P. acidovorans*	87
		17510	*P. testosteroni*	17
		17724	*P. palleronii*	7
	IIIb	17505	*P. delafieldii*	11
		11228	*P. facilis*	10
		19860	*P. alboprecipitans*	12
	IIIc	15946	*P. saccharophila*	6
	IIIx	17713	*A. paradoxus*	10
IV	IV	11426	*P. vesicularis*	2

[a] As Defined in Byng et al. (1980); Palleroni et al. (1973)
[b] Enzymological patterning groups delineated as in Byng et al. (1980), Whitaker et al. (1981a, 1981b)
[c] Generic abbreviations: *P.*, *Pseudomonas*; *A.*, *Alcaligenes*
[d] DNA homology values were determined using the S1 nuclease procedure of Johnson (1981)

P. acidovorans 11299a showed significantly less DNA homology with other subgroup members of pseudomonad Group III, and very little homology with representative species of pseudomonad Groups II and IV (Table 2).

Properties of DAHP synthase from P. acidovorans 11299a

DAHP synthase activity in crude extracts of *P. acidovorans* 11299a was tested for stimulation by divalent cations, a common feature of DAHP synthase from organisms within pseudomonad Groups I–IV (Whitaker et al. 1981a). Mn^{2+} was found to give greatest stimulation of DAHP synthase activity in crude extracts, whereas Co^{2+} stimulated activity to a slightly lesser extent (data not shown). Mg^{2+} had no effect on DAHP synthase activity. It is interesting to note that in partially purified DAHP synthase, Co^{2+} was found to give maximal stimulation, whereas Mn^{2+} became the less effective of the two metals. This phenomenon has been attributed to the presence in crude extracts of a cobalt-dependent 3-dehydroquinate synthase, the second enzyme of aromatic biosynthesis. Thus, when assaying DAHP synthase in crude extracts, cobalt stimulation may be partially masked as a result of product (DAHP) removal due to an enhancement of 3-dehydroquinate synthase activity by cobalt (Whitaker et al. 1982). The range of activation of DAHP synthase by Mn^{2+} varied depending on whether crude extracts were prepared in the presence or absence of 1.0 m*M* DTT, activity being stimulated 3.5-fold and 9-fold in extracts prepared with and without DTT, respectively. Table 3 shows that DAHP synthase activity in crude extracts of *P. acidovorans* 11299a has allosteric sensitivities similar to other members of subgroup IIIa. Whitaker et al. (1981a) obtained comparable results with other strains of subgroup IIIa and in fact observed the same general trends for the entire assemblage of pseudomonad Groups II and III.

Table 3. Comparative allostery of DAHP synthase in strains of representative species of pseudomonad Group IIIa

ATCC no.	Species	% Inhibition[a]			
		PHE	TYR	TRP	ARO
11299a	*Pseudomonas acidovorans*	21	48	0	79
17438	*P. acidovorans*	36	33	0	73
17409	*P. testosteroni*	19	39	0	59
11966	*P. testosteroni*	21	42	0	75
17724	*P. palleronii*	56	53	0	67

Crude extracts were prepared in 50 m*M* potassium phosphate buffer (pH 7.0) containing 1.0 m*M* DTT, and assays were carried out using standard reaction mixtures (see Methods). Mn^{2+} was found to give maximum stimulation of DAHP synthase in crude extracts of all sub-Group IIIa species tested and was therefore included in all assays. Specific activities usually ranged from 6.0 to 25.0 nanomol of product formed per minute per milligram protein
[a] Final concentration of each effector was 0.5 m*M*
Abbreviations: PHE, L-phenylalanine; TYR, L-tyrosine; TRP, L-tryptophan; ARO, L-phenylalanine + L-tyrosine + L-tryptophan. Other potential effectors tested (chorismate, prephenate, phenylpyruvate, and L-arogenate) had no effect on enzyme activity under these experimental conditions

Resolution of 2 isozymes of DAHP synthase

Data obtained from crude extracts of *P. acidovorans* 11299a were suggestive of the presence of isozymic forms of DAHP synthase. Figure 1 shows the DAHP synthase profiles obtained following fractionation of a crude extract of *P. acidovorans* 11299a by DEAE-cellulose chromatography. Panel A shows the profile obtained when crude extract was prepared and fractionated on DE-52 in the presence of 1.0 m*M* DTT. A very small but reproducible peak of activity was observed in the wash fractions. This peak of activity was found to be inhibited 89% by 0.5 m*M* L-phenylalanine, but was not inhibited by any of the other effector molecules tested (see legend to Table 4). A second peak of activity was recovered in the salt gradient (fraction no. 85). This activity was also inhibited by L-phenylalanine, 88% inhibition being achieved at 0.5 m*M* effector. In addition, a third peak of activity was found at fraction no. 105. This major peak of activity was strongly inhibited by L-tyrosine, with 0.1 m*M* L-tyrosine giving 100% inhibition.

The finding that the wash peak and early gradient peak (fraction no. 85) of DAHP synthase activity were both inhibited only by L-phenylalanine suggested the possibility that these peaks of activity could be two forms of a single isozyme. It was also noted that although these two activities were consistently present, the amount of activity in each peak varied between column runs. When an identical DEAE-cellulose column was run omitting DTT from buffer used for extract preparation and fractionation, the DAHP synthase profile in panel B of Fig. 1 was obtained. Under these conditions, no activity was recovered in the wash fractions, and the L-phenylalanine-regulated peak of activity at fraction no. 85 was elevated substantially. Although the L-tyrosine-regulated peak of activity eluted in the same posi-

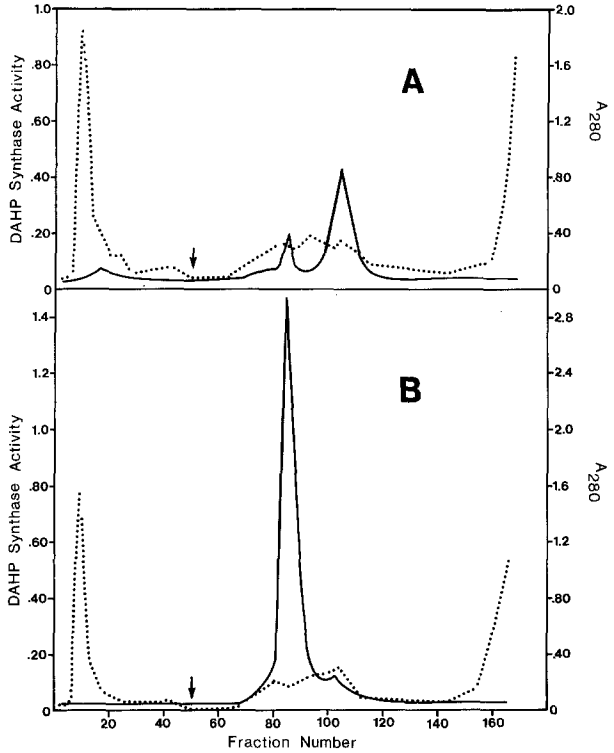

Fig. 1 A, B. DAHP synthase profiles obtained following fractionation of a crude extract of *Pseudomonas acidovorans* 11299a on DEAE-cellulose. DAHP synthase activity (solid line) represents absorbance at 549 nm. Dotted line indicates protein measured as A_{280}. Panel **A** represents the elution pattern observed when 1.0 m*M* DTT was included in buffers used throughout extract preparation and fractionation. The profile shown in panel **B** was obtained when DTT was omitted from buffers used. Arrows indicate starting point for the salt gradient (see Methods)

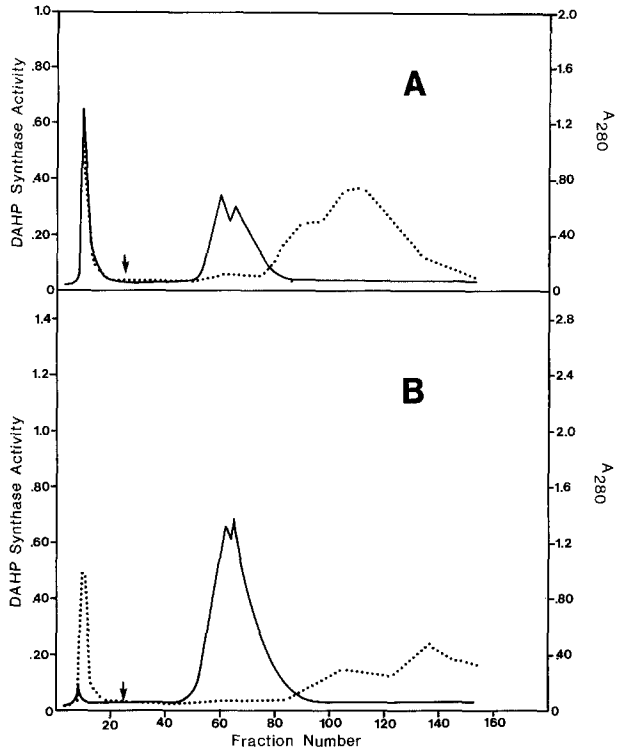

Fig. 2 A, B. Separation of DAHP synthase isozymes from *Pseudomonas acidovorans* 11299a via hydroxylapatite chromatography. Solid line represents DAHP synthase activity (measured as absorbance at 549 nm), dotted line indicates protein (measured as A_{280}). Panels **A** and **B** represent profiles obtained following fractionation of crude extracts prepared in the presence or absence of 1.0 m*M* DTT, respectively. Arrows indicate beginning of potassium phosphate gradient

tion, the activity recovered was decreased significantly in the absence of DTT.

The results of DEAE-cellulose chromatography were reproducible, and further experiments were carried out to examine more closely the effects of DTT on DAHP synthase activities. It was found that the specific activity of the L-phenylalanine-regulated peak of activity at fraction no. 85 of the DE-52 column run in the presence of DTT (Fig. 1, panel A) could be increased following dialysis to remove DTT. Conversely, when DTT was added to the L-phenylalanine-regulated peak of activity from a DE-52 column run in the absence of DTT (Fig. 1, panel B), activity was progressively lost (37% and 72% decreases in activity, at 13 and 36 h, respectively). An even more striking finding was that elution patterns of the L-phenylalanine-sensitive species could be altered when the individual peaks of activity were re-chromatographed on DEAE-cellulose in the presence or absence of DTT. For example, when the wash-peak fractions of DAHP synthase activity from a DE-52 column run in the presence of DTT were pooled, concentrated, passed through a Sephadex G-25 column to remove DTT, and re-chromatographed on a DE-52 column without DTT, two peaks of activity were seen. One peak of activity eluted in the wash fractions, and a second, larger peak of activity eluted in the salt gradient. On the other hand, when the single large L-phenylalanine-regulated peak of activity from a DE-52 column run in the absence of DTT was put in the presence of DTT (by means of a Sephadex G-25 column) and re-chromatographed on DE-52, two peaks of activity were again observed. As before, one peak of activity eluted in the wash, and a second peak of activity eluted in the salt gradient.

It was concluded that *P. acidovorans* 11299a possesses two isozymes of DAHP synthase, one regulated by L-phenylalanine and the other regulated by L-tyrosine (herein denoted DAHP synthase-phe and DAHP synthase-tyr, respectively). Since the two isozymes exhibited differential stabilities (i.e., response to DTT), each required different partial purification procedures prior to characterization. DAHP synthase-tyr was partially purified on DE-52 in the presence of DTT (see Fig. 1, panel A). On the other hand, even though DAHP synthase-phe was stable when partially purified on DE-52 (in the absence of DTT), this technique was not suitable for the separation and characterization of this isozyme due to the overlapping elution position with DAHP synthase-tyr (Fig. 1, panel B).

Hydroxylapatite was selected as a means of partially purifying DAHP synthase-phe for subsequent characterization. Figure 2 shows the elution profile of DAHP synthase from *P. acidovorans* 11299a when chromatographed on a Bio-Gel HTP column. The order of elution of the two DAHP synthase isozymes was the reverse of that observed using DE-52, and conditions were established (see Methods) such that DAHP synthase-tyr passed through the column

Table 4. Characterization of DAHP synthase isozymes from *Pseudomonas acidovorans* 11299a

Isozyme	$\bar{M}$[a]	pH optimum	$CoSO_4$ (mM)[b]	Inhibitory molecule[c]	Erythrose-4-phosphate[d]			Phosphoenolpyruvate[d]		
					K_m	K_i	Type of inhibition[e]	K_m	K_i	Type of inhibition[e]
DAHP synthase-tyr	251,000	7.0	0.02	L-Tyrosine	0.77	3.4 μM	C	0.26	1.7 μM	*N*
DAHP synthase-phe	67,000	6.8–7.2	–	L-Phenylalanine	0.87	25 μM	C	0.20	50 μM	*N*

[a] As determined by gel filtration chromatography (see Materials and methods)
[b] Concentration of $CoSO_4$ at which maximum stimulation of DAHP synthase-tyr was achieved. DAHP synthase-phe was not stimulated by any of the metal ions tested ($CoSO_4$, $MnSO_4$, $MgSO_4$)
[c] Screening for potential inhibitors was carried out at both saturating and sub-saturating ($\sim K_m$) concentrations of substrates. Compounds tested as possible inhibitors were: L-phenylalanine, L-tyrosine, L-tryptophan (each tested singly and in all possible combinations), chorismate, prephenate, phenylpyruvate, and L-arogenate. Each compound was tested at final concentrations of 0.1 and 0.5 mM
[d] K_m (mM) and V_m (nmol/min) were determined by plotting the reciprocal of velocity (v^{-1}) against reciprocal of the substrate concentration (mM^{-1}). $K_{m_{app}}$ and $V_{m_{app}}$ values were obtained from double reciprocal plots generated in the presence of inhibitor. These values were used to determine K_i by plotting [I] vs. $K_{m_{app}}/V_{m_{app}}$ (for competitive inhibition), and [I] vs. $1/V_{m_i}$ (for non-competitive inhibition)
[e] C = competitive, N = non-competitive

unretarded, whereas DAHP synthase-phe eluted in the potassium phosphate gradient. Panels A and B of Fig. 2 show DAHP synthase profiles obtained in the presence and absence of DTT, respectively. Again it was observed that DAHP synthase-tyr required DTT for stability, whereas DAHP synthase-phe activity was significantly reduced by the addition of DTT. On hydroxylapatite, the elution pattern of DAHP synthase-phe did not change in the presence of DTT, as occurred on DE-52. However, for reasons yet unclear, DAHP synthase-phe did not elute as a sharp peak of activity on hydroxylapatite, either in the presence or absence of DTT. The possibility that this peak contains two separate isozymes seems unlikely since fractions throughout the peak exhibited the same degree of sensitivity of L-phenylalanine (data not shown). Therefore, DAHP synthase-phe partially purified by hydroxylapatite chromatography (in the absence of DTT) was used for further characterization.

Characterization of DAHP synthase-tyr

A summary of the enzymological characterization of DAHP synthase-tyr is shown in Table 4. The isozyme has an apparent molecular weight of 251,000. A pH optimum of 7.0 was determined using a series of 0.5 M potassium phosphate buffers (ranging from pH 6.4 to 7.8). Co^{2+} provided greatest activation of any metal ion tested, with maximum stimulation occurring at 0.02 mM $CoSO_4$. All subsequent kinetic analyses were carried out at pH 7.0, under conditions of maximal cobalt activation. K_m values for E4P and PEP, (determined by Lineweaver-Burke plots), were 0.77 mM and 0.26 mM, respectively. Inhibition by L-tyrosine was competitive with respect to E4P (K_i = 3.4 μM) and non-competitive with respect to PEP (K_i = 1.7 μM).

Characterization of DAHP synthase-phe

The molecular weight of DAHP synthase-phe was estimated to be 67,000 (see Table 4). It should be noted, however, that the DAHP synthase-phe activity recovered following gel filtration was very low; drastically reduced from the amount of activity loaded onto the gel. Therefore, the possibility cannot be excluded that DAHP synthase-phe is separable by gel filtration into different molecular weight species, with only one form being detectable under conditions used. This would indeed be consistent with results obtained following DE-52 chromatography in the presence of DTT (see Fig. 1, panel A). DAHP synthase-phe exhibited a broad pH optimum (6.8–7.2) and pH 7.0 was used for further characterization. Unlike DAHP synthase-tyr, DAHP synthase-phe activity was not stimulated by any of the divalent cations tested. K_m values determined for E4P and PEP were 0.87 mM and 0.20 mM, respectively. L-Phenylalanine inhibited competitively with respect to E4P (K_i = 25 μM) and non-competitively with respect to PEP (K_i = 50 μM).

Discussion

Phylogenetic status of Pseudomonas acidovorans 11299a

Enzymological patterning of aromatic amino acid biosynthesis clearly implicated strain ATCC 11299a as a Group IIIa pseudomonad. DNA homology studies confirmed this placement, and further established this organism's species identity as *acidovorans*. The definitive taxonomic placement of *P. acidovorans* 11299a described here suggests further avenues of inquiry regarding the presence of phenylalanine hydroxylase in organisms phylogenetically near Group III pseudomonads, and indeed within Group III.

Phenylalanine hydroxylase

The presence of phenylalanine hydroxylase has been documented in only a few prokaryotes. However, this presumably catabolic enzyme may exist in a larger number of organisms capable of utilizing L-phenylalanine as a sole source of carbon and energy (although the ability to grow on L-phenylalanine is not necessarily indicative of phenylalanine hydroxylase activity). The presence of phenylalanine hydroxylase in *P. facilis* (DeCicco and Umbreit 1964), a

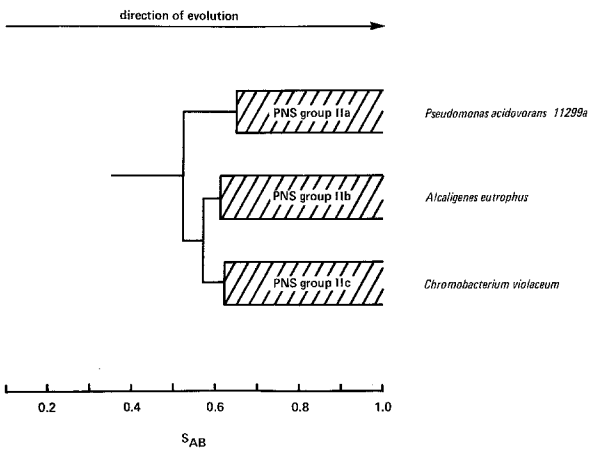

Fig. 3. Simplified dendrogram showing the phylogenetic relationships between members of the purple non-sulfur (PNS) Group II established via oligonucleotide cataloging (Fox et al. 1980; Stackebrandt and Woese 1981). S_{AB} values represent association coefficients which provide a quantitative expression of phylogenetic relatedness (Fox et al. 1980). Representative species known to possess phenylalanine hydroxylase are listed at the right of each grouping. Other members of each subgroup are as follows (Stackebrandt and Woese 1981): PNS IIa; *Rhodopseudomonas gelatinosa*, *Rhodospirillum tenue*, *Sphaerotilus natans*, *Pseudomonas testosteroni*, *Comamonas terrigena*, and *Aquaspirillum gracile*. PNS IIb; *Chromobacterium lividum*, *Pseudomonas cepacia*, and *Alcaligenes faecalis*. PNS IIc; *Aquaspirillum serpens*

Group IIIb pseudomonad (Whitaker et al. 1981b), and *Alcaligenes eutrophus* (Friedrich and Schlegel 1972), a member of Group II (Byng et al. 1983b), suggests that this enzyme could be generally distributed throughout both of these groups. Fox et al. (1980) used oligonucleotide cataloging to establish three major phylogenetic groups among the purple photosynthetic bacteria and their relatives. One of these groups, termed the purple non-sulfur-2 group (Fox et al. 1980) or Superfamily A (Byng et al. 1983b), can be represented by the simplified dendrogram shown in Fig. 3. These groupings support the possibility that phenylalanine hydroxylase is conserved throughout Superfamily A (Fig. 3). For example, PNS Group IIa contains *P. acidovorans* (and interestingly, *C. terrigena*), PNS Group IIb contains *A. eutrophus*, and finally, PNS Group IIc includes *Chromobacterium violaceum* (which is known to possess phenylalanine hydroxylase [Letendre et al. 1974]). Thus, each subgroup within Superfamily A contains at least one organism that exhibits phenylalanine hydroxylase activity.

However, the alternative possibility cannot be excluded that phenylalanine hydroxylase activity may be a wide-ranging characteristic that is scattered throughout a variety of organisms. Location of the gene for phenylalanine hydroxylase on a wide host-range plasmid would be consistent with this idea, and would also be in keeping with the probable dispensability of a gene encoding a catabolic capability such as L-phenylalanine utilization.

DAHP synthase in pseudomonad Groups II and III

Member species of pseudomonad Groups II and III were reported (Whitaker et al. 1981a) to share a general sensitivity to feedback inhibition by either L-tyrosine or L-phenylalanine. Quantitative differences seemed to exist between the two groups when combinations of the two inhibitors were tested. The latter studies were carried out with crude extracts prepared in the presence of DTT. In light of our findings about the effects of DTT on DAHP synthase isozymes from *P. acidovorans* 11299a, the apparent differences between Groups II and III may reflect a general difference in DTT effects rather than any basic difference in isozyme pattern.

Our results demonstrate that *P. acidovorans* 11299a possesses two allosteric isozymes of DAHP synthase, and data obtained from other Group III pseudomonads (Whitaker et al. 1981a) indicate that these isozymes are conserved throughout Group III (and probably Group II). It is difficult to ascertain the quantitative impact of each isozyme due to differential stabilities during extract preparation. DAHP synthase-tyr appears to require DTT only for stabilization. DAHP synthase-phe on the other hand, exhibits a more complicated response to DTT. One possibility is that DTT decreases the activity of DAHP synthase-phe by effectively reducing one or more disulfide linkages in the enzyme molecule. The resulting conformational change not only affects the catalytic activity of the enzyme, but also alters the ionic character of the molecule, thus giving rise to variation in elution patterns observed following ion-exchange chromatography. Both positive and negative effects of DTT on the activity of enzymes involved in photosynthesis have been recently reviewed (Buchanan 1980), and differential responses to this compound have been noted in our laboratory for several enzymes within the aromatic amino acid pathway in *Xanthomonas campestris* and *Erwinia* species (unpublished observations).

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